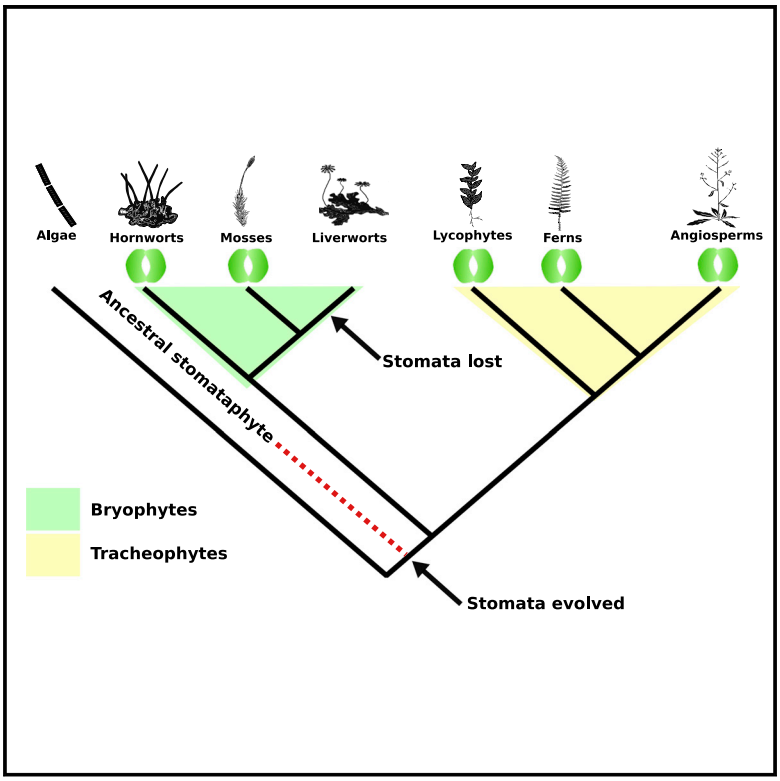
Article

Current Biology

Phylogenomic Evidence for the Monophyly of Bryophytes and the Reductive Evolution of Stomata

Graphical Abstract



Authors

Brogan J. Harris, C. Jill Harrison, Alistair M. Hetherington, Tom A. Williams

Correspondence

tom.a.williams@bristol.ac.uk

In Brief

Harris et al. provide phylogenomic support for the monophyly of bryophytes and show that many of the genes that pattern and operate stomata in modern tracheophytes, such as *Arabidopsis*, were already present in the common ancestor of land plants. The analyses indicate that the simple stomata of modern bryophytes are a result of reductive evolution.

Highlights

- Land plants comprise two sister lineages, bryophytes and tracheophytes
- Their common ancestor possessed complex stomata
- Stomata were lost or reduced during the evolution of bryophytes
- Liverwort air pores evolved following stomatal loss in the liverwort ancestor

Harris et al., 2020, Current Biology *30*, 2001–2012
June 8, 2020
https://doi.org/10.1016/j.cub.2020.03.048

CellPress

Current Biology

CellPress
OPEN ACCESS

Article

Phylogenomic Evidence for the Monophyly of Bryophytes and the Reductive Evolution of Stomata

Brogan J. Harris,[1] C. Jill Harrison,[1] Alistair M. Hetherington,[1] and Tom A. Williams[1,2,*]
[1]School of Biological Sciences, University of Bristol, Life Sciences Building, 24 Tyndall Avenue, Bristol BS8 1TQ, UK
[2]Lead Contact
*Correspondence: tom.a.williams@bristol.ac.uk
https://doi.org/10.1016/j.cub.2020.03.048

SUMMARY

The origin of land plants was accompanied by new adaptations to life on land, including the evolution of stomata—pores on the surface of plants that regulate gas exchange. The genes that underpin the development and function of stomata have been extensively studied in model angiosperms, such as *Arabidopsis.* However, little is known about stomata in bryophytes, and their evolutionary origins and ancestral function remain poorly understood. Here, we resolve the position of bryophytes in the land plant tree and investigate the evolutionary origins of genes that specify stomatal development and function. Our analyses recover bryophyte monophyly and demonstrate that the guard cell toolkit is more ancient than has been appreciated previously. We show that a range of core guard cell genes, including SPCH/MUTE, SMF, and FAMA, map back to the common ancestor of embryophytes or even earlier. These analyses suggest that the first embryophytes possessed stomata that were more sophisticated than previously envisioned and that the stomata of bryophytes have undergone reductive evolution, including their complete loss from liverworts.

INTRODUCTION

The colonization of land by plants was a foundational event in the history of life on Earth, opening up entirely new niches for the diversification of terrestrial life and permanently changing the carbon cycle [1]. Land plants (embryophytes) are a monophyletic lineage that evolved from within freshwater streptophyte algae, radiating c. 470–515 mya [2, 3]. Recent work suggests that their closest living relatives among streptophytes are the Zygnematales [2, 4, 5], including filamentous forms, such as *Spirogyra*. The conquest of land involved adaptations to drier and more variable environments and, as plants diversified and became larger, subsequently involved the evolution of transport systems and more efficient means of resource capture [6, 7]. One pivotal adaptation of early land plants was stomata, microscopic valves that occur on the aerial tissues of most land plants. Following the evolution of the cuticle, stomata enabled the entry of carbon dioxide and the exit of water vapor, allowing plants to exist in more arid and variable conditions [1]. Although the development and function of stomata in the model angiosperm *Arabidopsis thaliana* are well described (Figure S2), the evolutionary origins and ancestral function of stomata remain less well understood [8–12].

Distinguishing between alternative hypotheses of stomatal evolution is challenging for two main reasons. First, not all land plants possess stomata [11], and when present, stomatal function and morphology vary substantially, from single binucleate stomata in *Funaria* [13] to stomata with subsidiary cells in horsetails [14] and kidney-shaped stomata in flowering plants, such as *Arabidopsis* [15]. Second, the evolutionary relationships among modern land plants, and the positions of the stomata-bearing and lacking lineages, remain uncertain.

Long-standing phylogenetic hypotheses place bryophytes (mosses, liverworts, and hornworts) as a grade at the base of vascular plants (tracheophytes), a group containing flowering plants (angiosperms), gymnosperms, ferns, horsetails, and lycophytes [6, 16]. By contrast, recent analyses suggest that mosses and liverworts form a sister clade to all other embryophytes [2, 17] or that bryophytes form a monophyletic sister group to tracheophytes [18, 19]. These alternative phylogenetic hypotheses are central to testing hypotheses of stomatal evolution because liverworts and some mosses lack stomata. Stomata could have been gained once in the common ancestor of land plants and then lost [8, 20, 21], or alternatively, they might have been gained several times independently [11, 22, 23]. Here, we set out to resolve the phylogeny of land plants, determine the evolutionary history of genes that underpin stomatal development and function, and infer the nature of the first stomata.

RESULTS

The recent publication of new genomes and transcriptomes from the 1KP project [19], including an improved sampling of key bryophyte lineages, provides an opportunity to re-evaluate the best supported phylogeny of land plants. We used the orthology inference tool OrthoFinder [24] in combination with manual gene family curation (see STAR Methods) to identify 151 single-copy orthologs from 162 Viridiplantae genomes and transcriptomes, including 143 land plants and 19 algal outgroups. Analysis of the concatenated alignment under the best-fitting LG+C60+G+F

model in IQ-Tree [25] resulted in the topology shown in Figure 1, in which bryophytes were monophyletic with maximal support (100% bootstrap). Zygnematales branched sister to embryophytes, consistent with previous findings [2, 4, 5] (reviewed in [6, 12]).

Bryophyte monophyly demands a reappraisal of early stomatal evolution because it supports the hypothesis that all modern stomata are homologous; that is, stomata evolved once in the common ancestor of land plants, and liverworts and some mosses later lost them secondarily [23]. Based on gene expression analyses [26, 27] and a review of the literature [21] (Data S1I), we identified a set of genes that are implicated in stomatal development (14 genes) and function (18 genes)—a guard cell "toolkit" (see STAR Methods). Many of the genes in the guard cell toolkit belong to large multigene families. In investigating their origins, key questions relate to the conservation of orthologs, not just homologs, of the *Arabidopsis* genes across the embryophyte tree.

Orthologs are homologous genes that diverged at a speciation event [28, 29], such as the TMM and TPK1 genes of *Arabidopsis* and *Physcomitrella patens* (Figure 2). Paralogs are genes that diverged at a duplication event, such as the ABI1 and MYB60 genes of *Arabidopsis* (Figure 2). Gene or genome duplication can give rise to a situation in which two or more genes in one species are orthologous to a single gene in an outgroup species, such as *PHOT1* and *PHOT2* in *Arabidopsis* to the single *PHOT* gene in *Physcomitrella*; these *Arabidopsis* genes are co-orthologs of the *Physcomitrella* gene. This evolutionary perspective is useful because orthologs are more likely than co-orthologs and paralogs to have conserved functions [30], enabling us to draw more reliable inferences about gene function across the embryophyte tree. That said, independent histories of gene duplication and loss in different lineages lead to a spectrum of relationships among gene family members in embryophytes, and so, in what follows, we interpret gene tree topologies and their implications for shared functions conservatively. To identify orthologs of the *Arabidopsis* guard cell toolkit in other embryophytes, we inferred gene family phylogenies for each toolkit member and then inferred gene origins, duplications, losses, and orthology relationships by comparison to the species tree (Figure 1), using the approach illustrated in Figure 2.

Phylogenetic analyses suggested that orthologs of 7 out of the 14 genes involved in stomatal development were present in the last common ancestor of embryophytes (Figure 3A). Moreover, an additional four gene families were predicted to have a single orthologous representative in that common ancestor but, due to duplications in the angiosperm lineage, are represented by two co-orthologous genes in *Arabidopsis* (Figure 2A), where the majority of experimental characterization has been carried out. For these families, the inference of shared function in the ancestor is weaker because gene duplications are sometimes followed by functional divergence [30]. For example, our analyses indicate that *ERECTA* was present as a single copy in the embryophyte ancestor; a gene duplication in the stem lineage of angiosperms then gave rise to *ERECTA* and *ERL1+2*, with a subsequent duplication giving rise to *ERL1* and *ERL2*. *Arabidopsis ERL1, 2* and *ERECTA* are collectively co-orthologous to a set of *ERECTA* genes [31] in *Physcomitrella* and other bryophytes that emerged from bryophyte-specific duplications (Figure S11; see supplemental data on FigShare). Overall, the results suggest that there has been extensive secondary loss of stomatal developmental orthologs during the diversification of land plants.

The basic-helix-loop-helix (bHLH) family of proteins, specifically SPCH, MUTE, and FAMA, governs stomatal development in angiosperms (Figure 6A). Analyses of bHLH gene family evolution have identified *SPCH*, *MUTE*, and *FAMA* homologs in bryophytes and have suggested that these sequences form a clade within the broader bHLH evolutionary tree [21]. Our updated bHLH phylogeny (Figure 4) resolves the relationships within that clade to suggest that the common ancestor of embryophytes already possessed *SMF*, *FAMA*, and a single gene ancestral to both *SPCH* and *MUTE* (*SPCH*/*MUTE*). This inference is supported by our phylogenetic analysis and the presence, in modern lycophytes, of orthologs of both SMF and FAMA (Figure 4). This single gene duplicated in the angiosperm stem lineage after divergence from gymnosperms to form *SPCH* and *MUTE*. These duplications may have been associated with functional divergence, as reported for the *KNOX* genes of land plants (reviewed in [6]). Considerable lineage-specific losses then ensued: *SMF* was lost in most tracheophyte lineages and *FAMA* and *SPCH*/*MUTE* were lost in all bryophytes. As some branches in the bHLH tree had low or moderate bootstrap support, we tested the alternative hypothesis that *FAMA*, *SPCH*, and *MUTE* evolved from *SMF* following the bryophyte/tracheophyte divergence using an approximately unbiased (AU) test; this alternative was rejected (AU = 0.009).

The most conserved guard cell toolkit gene is *SCRM/2*, with orthologs detected in all lineages except two highly derived liverworts, *Monoclea gottschei* and *Riccia berychiana* (Figure 3A). The *Physcomitrella* SCRM protein has been shown to interact with SMF and to function in stomatal development [9]. Two paralogous genes in *Arabidopsis*, *SCRM1* and *SCRM2*, evolved recently (~17.3 mya) from this single-copy form via a gene duplication [32]; all other plants surveyed, including those without stomata, possess at least one copy of the gene, with some having experienced lineage-specific duplications. This ubiquitous presence of *SCRM/2* may be due to an alternative role it plays in the plant cell; one possibility is a role in cold tolerance [33], although the genetic link to this process has recently been challenged [34]. The hypothesized stomatal development pathway for the common ancestor of extant embryophytes is reconstructed (Figure 6C), using the pattern of presence and absence of orthologs in Figure 3A.

Lineage-specific loss was also observed in the EPF family of proteins (Figure S4A). Orthologs of EPF1 were identified in *Physcomitrella* and *Selaginella moellendorffii*, which suggests that the duplication that generated EPF1 and EPF2 also pre-dated the divergence of bryophytes and tracheophytes. Bootstrap support for the EPF1 clade was 60%, although EPF2 received stronger support at 81%. The phylogeny of mature cleaved EPF1 and EPF2 also supported the conclusion of the full-length sequence analyses, albeit with lower bootstrap support (61% and 63%, respectively; Figure S4B). The maximum likelihood tree is consistent with the presence of both *EPF1* and *EPF2* genes in the embryophyte ancestor, with subsequent loss of *EPF2* in all lineages other than angiosperms and *EPF1* in most bryophytes and some early-diverging tracheophytes (this scenario is depicted in Figure 3). However, bootstrap support for the key

CellPress
OPEN ACCESS

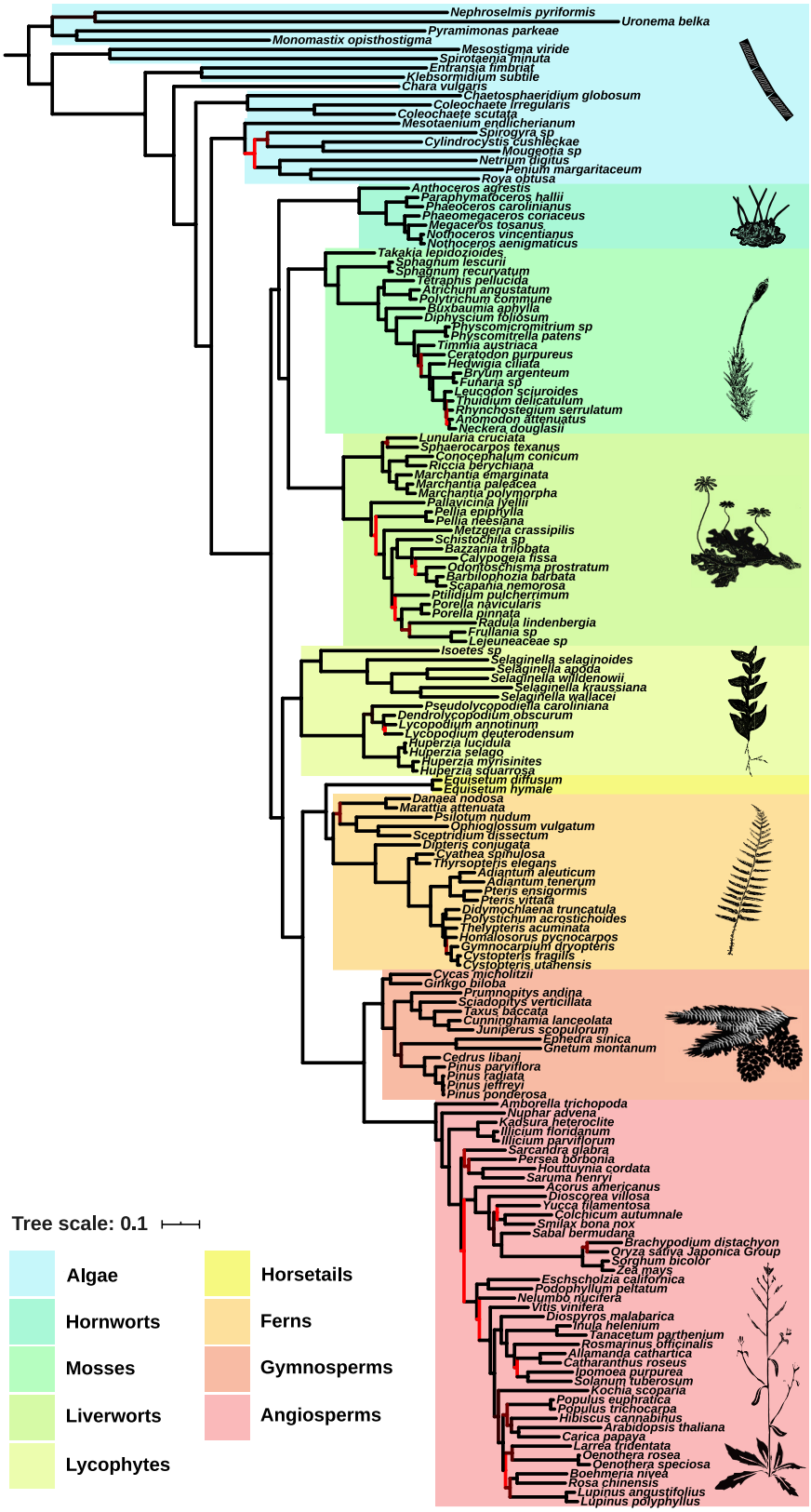


Figure 1. A Species Tree of Land Plants and Their Algal Relatives Provides Robust Support for Bryophyte Monophyly

The maximum likelihood tree was inferred from a concatenation of 151 orthologs conserved across 162 Viridiplantae genomes and transcriptomes using IQ-Tree [25]. The Bayesian information criterion (BIC) was used to select the best-fitting substitution model (LG+C60+G+F). All branches that did not receive 100% bootstrap support are indicated with red branches—full tree file is provided in the data supplement.

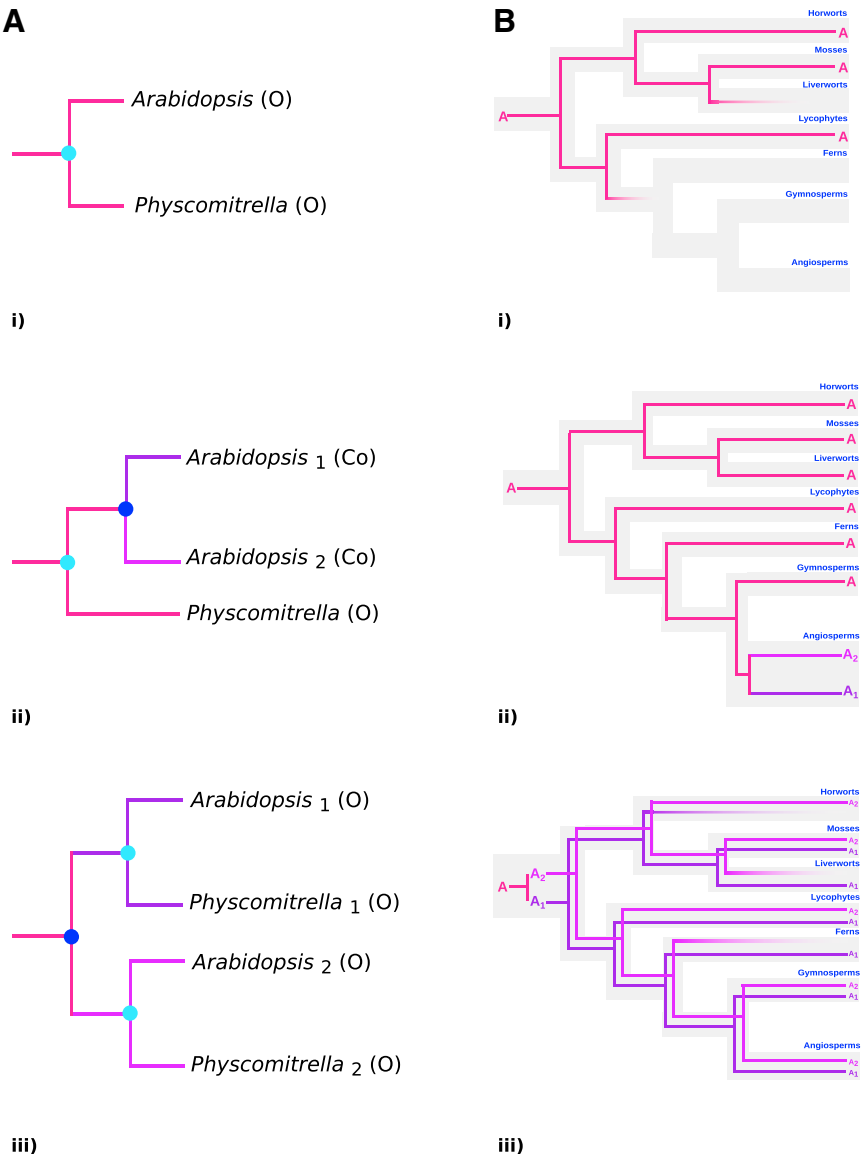


Figure 2. Tracing the Evolution of Stomatal Toolkit Genes

(A) Relations among homologous genes in modern plant species. (Co), co-ortholog; (O), ortholog. (i) A single-copy ortholog conserved between *Arabidopsis* and *Physcomitrella* is shown. (ii) Due to a duplication in the *Arabidopsis* lineage, the two *Arabidopsis* genes are co-orthologous to a single gene in *Physcomitrella*. (iii) Due to a duplication prior to the divergence of bryophytes and tracheophytes, two paralogous gene families are conserved in *Arabidopsis* and *Physcomitrella*; within each paralogous clade, single-copy orthologs are conserved between *Arabidopsis* and *Physcomitrella*. In real data, independent gene losses can obscure these relationships, and single-gene phylogenies can be used to distinguish orthology from paralogy. Dark-blue circles indicate duplication events, and light-blue circles indicate speciation events.

(B) Gene family origins on the embryophyte tree. (i) A single-copy ortholog is present in lycophytes and bryophytes; drawing the gene tree into the species tree reveals a secondary loss in euphyllophytes. (ii) A single-copy ortholog is conserved across embryophytes but was duplicated in the angiosperm stem lineage, leading to a two-to-one co-orthologous relationship between the angiosperm and other embryophyte sequences. (iii) In many families, we observe a more complex evolutionary history involving multiple duplications and independent losses.

branches is low to moderate, and an AU test could not reject the alternative scenario in which *EPF1* and *EPF2* arose from a tracheophyte-specific duplication following the divergence of bryophytes and tracheophytes (AU = 0.813). Under this scenario, the *EPF* gene in the embryophyte common ancestor would instead represent a pre-duplication ortholog of *Arabidopsis EPF1/2*.

We also observed losses of *EPF1*, *EPF2*, and *TMM* in two tracheophytes that have secondarily lost stomata, namely the angiosperm *Zostera marina*, which has previously been reported to have lost these and other stomatal development genes [35] and the lycophyte *Isoëtes tegetiformans* (Data S1A). As EPF1/2 and TMM are negative regulators of stomatal development, their loss during stomatal reduction likely occurred subsequent to the deletion of positive regulators. Consistent with this view, double mutants of *epf1 and tmm* in *Physcomitrella* did not lead a decrease in the number of stomata on the sporophyte capsule [31], suggesting that the deletion of genes that promote stomatal development likely represents the first stage of stomatal loss. Interestingly, *EPF1/2* and *TMM* were the only genes lost in both *Isoëtes* and *Zostera*, indicating that the loss of stomata in these distinct lineages was associated with a loss of different positive regulators.

Next, we investigated the origins of genes involved in stomatal function in *Arabidopsis*. Our analysis suggested that only 6 out of the 18 functional components of the guard cell toolkit had orthologs in the embryophyte common ancestor (Figure 3B). Although homologs of several additional components (*ABI1*, *PHOT1*, and *HT1*) have been described in *Physcomitrella* [36], our analysis indicates that these gene families have undergone duplications in the angiosperm stem lineage (*ABI1*) and gymnosperm/angiosperm stem lineage (*PHOT1* and *HT1*), respectively. As gene duplication is often associated with functional change [30], it is difficult to ascribe the functions of these *Arabidopsis* genes to the ancestral stomataphyte, despite the presence of gene family members ("pre-duplication" orthologs; see Figure 3) in both bryophytes and tracheophytes. In the tracheophytes, 11 out of the 18 functional genes in the toolkit have undergone duplications, and all of these genes have a single conserved homolog from the same gene family dating back to the root of embryophytes (Data S1A).

Phylogenetic analysis of stomatal function genes has recently been undertaken by Sussmilch et al. [37]. Their analysis of the

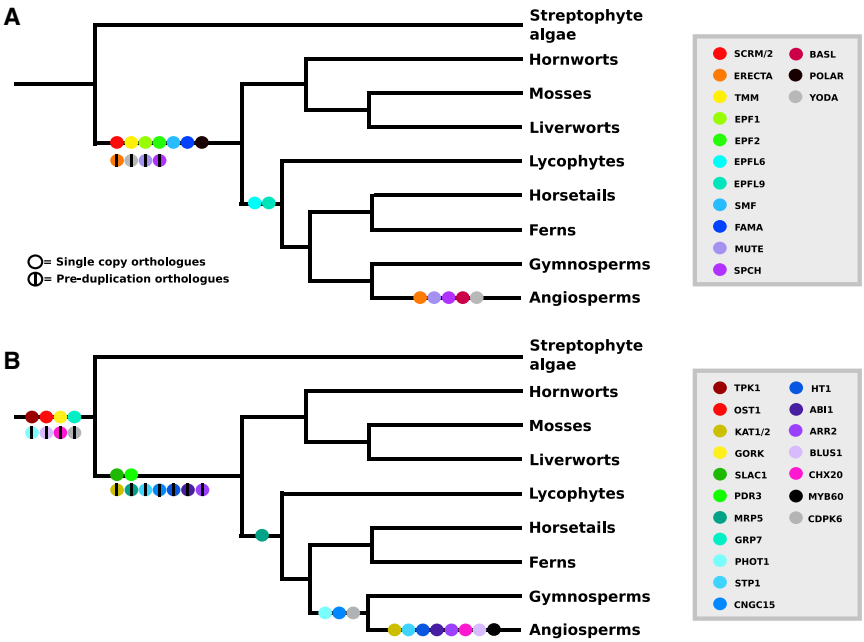


Figure 3. The Presence and Absence of Orthologous Genes Involved in Stomatal Development and Function

(A and B) Single-copy orthologs (solid circles) are conserved as a single gene in modern plants and likely perform the same function. Pre-duplication orthologs (striped circles) are genes that were present in a single copy in the embryophyte ancestor but were later duplicated in some (but not all) lineages; a number of characterized stomatal toolkit genes in *Arabidopsis* fall into this category. Single-copy orthologs of 6/18 genes involved in stomatal function (B) and 7/14 genes involved in stomatal development (A) were already present in the embryophyte common ancestor. Pre-duplication orthologs of an additional 11 functional and 4 developmental genes were also present in the stem lineage of land plants, although some of their stomata-specific functions may have evolved subsequent to gene duplication in the angiosperm or spermatophyte lineages. See also Figure S2 and Data S1A–S1F.

light signaling kinase *BLUS1* suggested that the gene originated in the common ancestor of angiosperms. Our results agree that *BLUS1* originated in a gene duplication in the angiosperm stem lineage but also highlight that *BLUS1* has a closely related and highly conserved pre-duplication ortholog dating back to the streptophyte algae (Figure S3A). BLUS1 interacts with the phototropin PHOT1 in blue light signaling [38]. Interestingly, *PHOT1* exhibits a similar phylogeny to *BLUS1*, with a duplication in the ancestor of angiosperms leading to *PHOT1* and *PHOT2*, which are co-orthologous to a single gene in other embryophytes (Figure S3B). The single phototropin in *Marchantia polymorpha*, which is an ortholog of *PHOT1/2* (Figure S3B), has been found to partially rescue the function of an *Arabidopsis phot1/phot2* mutant and thus act as a general photoreceptor [39]. Thus, it is possible that the ortholog of BLUS1 also performs a less specific signaling role in non-angiosperm embryophytes (Figure S3A).

Orthologs of guard cell ABA signaling pathway genes date back to the ancestor of embryophytes (*PDR3* and *SLAC1*) or earlier in archaeplastid evolution (*OST1* and *GORK*; Figure 3B). Moreover, phylogenies of additional components of the pathway, such as *ABI1* and *CDPK6*, highlight the presence of conserved orthologs in the ancestral lineage. The presence of these genes might suggest that the ability to control guard cell turgor was present in the ancestral embryophyte.

In order to maximize taxon sampling across the plant tree, many (162 of 175) of the datasets we analyzed are transcriptomes rather than complete genomes. Ortholog absence from transcriptome data does not imply absence from genomes but could instead reflect a lack of expression, and this effect could inflate the inferred rate of secondary loss near the tips of the tree. To investigate discrepancies between genome and transcriptome data, we performed a complementary analysis solely using predicted protein sequences from complete genomes (Data S1C and S1F). The results were consistent with our full analysis, with 6/14 development genes and 8/18 functional genes already predicted to be present at the embryophyte root (Data S1C). We also observed the same numbers of gene losses in *Marchantia polymorpha* for both the genome only and full analyses for both development genes (5 lost) and functional genes (2 lost). To complement our manually curated gene tree and ortholog inferences, we repeated our analyses using the automated ortholog inference method OrthoFinder [24] (Supplemental Information).

DISCUSSION

Our analyses provide additional support for the hypothesis of bryophyte monophyly [3, 18, 19, 40] and suggest that the stomata of bryophytes and some early-diverging tracheophytes evolved by secondary reduction from an ancestral form with higher gene content through loss of genes controlling development and function. Our results are consistent with the hypothesis that the stomata of the ancestral embryophyte were more similar to those of modern tracheophytes than bryophytes [2, 12], specifically with the reconstruction of the ancestral development pathway being more similar to *Arabidopsis* than *Physcomitrella* (Figure 5). The reconstruction suggests that some of the genes that govern one cell spacing (a developmental process that separates stomata by at least one cell) [41] and epidermal patterning pathways of *Arabidopsis* were present in the ancestral embryophyte (Figure 6C) and are conserved throughout the evolutionary history of land plants, corroborating the findings of Chater et al. [21] and Caine et al. [31]. In addition to the presence of *TMM*, *ERECTA*, and *EPF1* in the ancestor of embryophytes, our reconstructions suggest that *EPF2*, *SPCH*/*MUTE*, *FAMA*, and *POLAR* also date back to the last common ancestor of embryophytes and, therefore, that the stomata of *Physcomitrella* evolved by reduction from a more tracheophyte-like ancestor. Our results are consistent with the hypothesis that the embryophyte common ancestor already possessed actively controlled stomata,

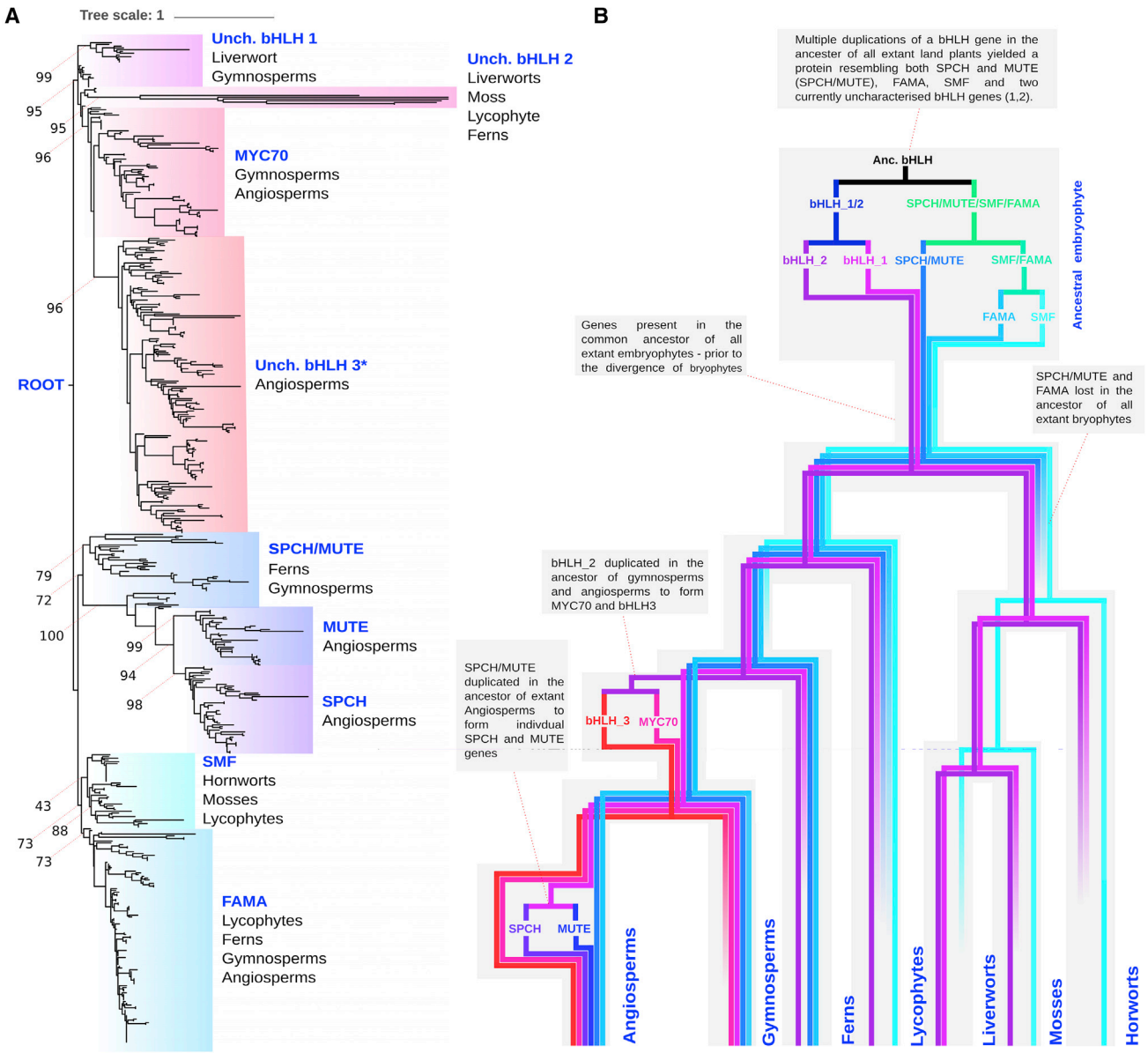


Figure 4. Rooted Phylogenetic Gene Tree for the bHLH Family of Genes, Including *SPCH*, *MUTE*, and *FAMA*

(A) A maximum likelihood tree rooted on a gene duplication in the bHLH family that likely pre-dated the divergence of bryophytes and tracheophytes, based on the presence of both clades on either side of the root. By the same reasoning, the root of the SMF/FAMA clade maps at least to the embryophyte root, and so the gene duplication giving rise to the *SMF/FAMA* and *SPCH/MUTE* families must also pre-date the divergence of embryophytes. This suggests that *SPCH/MUTE* has been secondarily lost in bryophytes. The duplication of *SPCH/MUTE* to form *SPCH* and *MUTE* occurred in angiosperms after their divergence from gymnosperms. The presence of lycophyte sequences within the *SMF* clade is the key data point, suggesting that *SMF* dates back to the ancestor of all embryophytes (and not just bryophytes), with the same being true for duplicated sister clade *FAMA* (branch has 88% bootstrap support). Thus, the phylogeny of the bHLH family suggests that the last common ancestor of embryophytes had an ortholog of *SMF*, *FAMA*, and a protein that resembles both SPCH and MUTE (SPCH/MUTE). *, multiple bHLH genes present. Bootstrap support values for key branches are noted.

(B) Graphical representation of the logic used to determine history of the gene family; faded lines represent gene loss.

with key regulators of the stomatal closure signaling pathway in response to ABA pre-dating the divergence between tracheophytes and bryophytes (Figure 3) [20]. It has been proposed that this reductive evolution may not be unique to guard cells but may also have occurred in other elements of bryophyte biology, such as in the evolution of the vascular system [12].

It is worth noting that our evolutionary reconstruction of ancestral stomata is likely incomplete, because we are limited by what is known about the genes that specify stomata in angiosperms, particularly *Arabidopsis.* Genes that are not found in angiosperms but that specify stomata in other plant lineages are therefore absent from our reconstruction. The variable distribution of

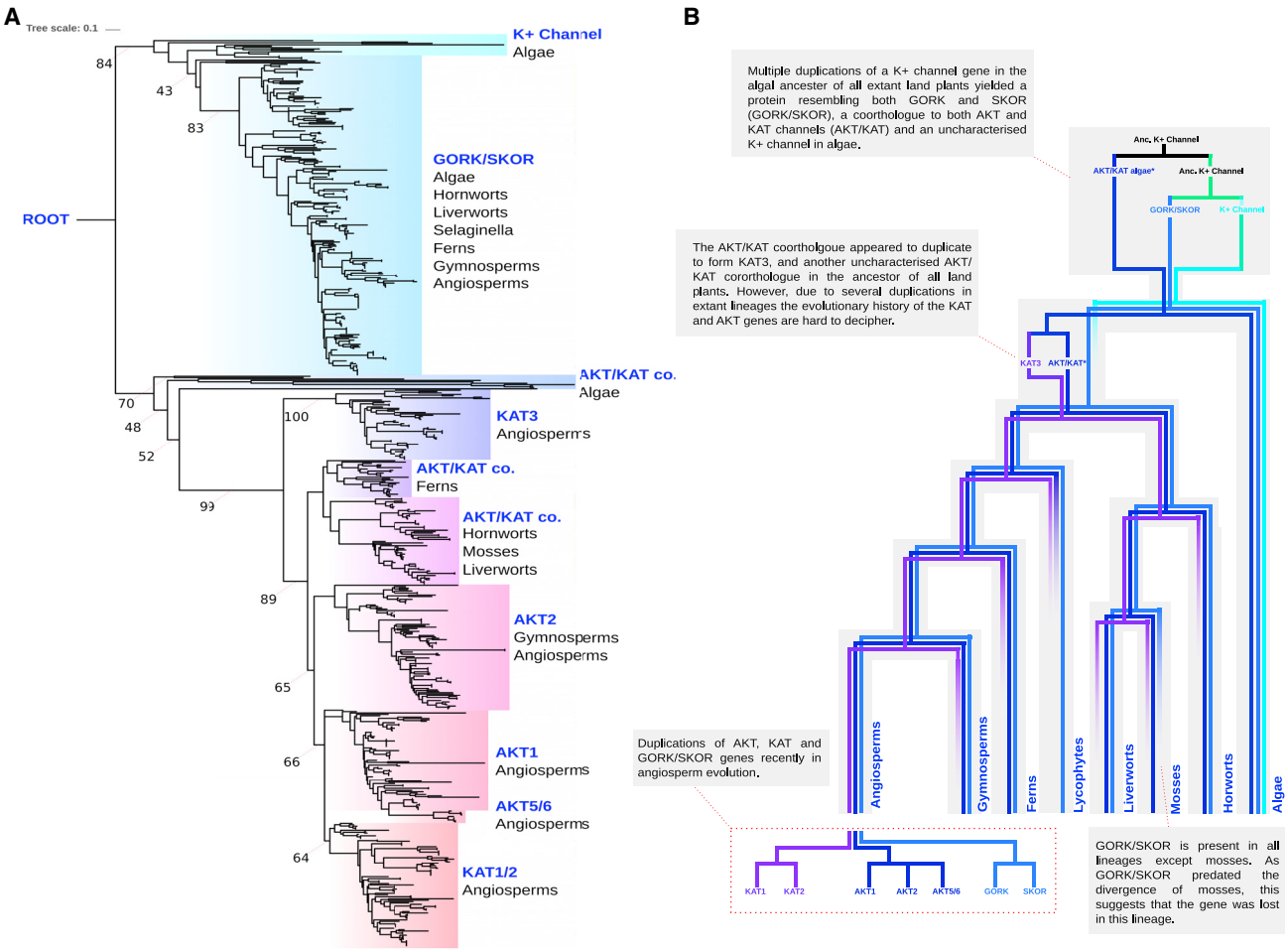


Figure 5. Rooted Phylogenetic Gene Tree for the *SHAKER* Family of Genes, Including GORK, SKOR, KAT, and AKT Ion Channels

(A) The tree is rooted on a gene duplication that pre-dated the divergence of embryophytes from their closest algal relatives, giving rise to the *GORK/SKOR* and *KAT/AKT* gene families. *GORK* and *SKOR* arose from a recent *Brassica*-specific gene duplication, and most embryophytes (including angiosperms) have a single gene, here called *GORK/SKOR*. Close relatives of *Brassicas* have functioning stomata but only have the single copy (GORK/SKOR), suggesting that the protein was already integral for stomata function prior to the duplication. Sequenced mosses do not possess *GORK/SKOR* orthologs; given the presence of liverwort sequences in the *GORK/SKOR* clade, this implies that the *GORK/SKOR* gene was lost secondarily in mosses. Note that, although the maximum likelihood tree places *KAT3* at the base of the radiation of *AKT/KAT* ion channels, an alternative position sister to *KAT1/2* was not rejected by AU test (AU = 0.166).

(B) Graphical representation of the logic to determine gene family history; faded lines represent gene loss.

orthologs across stomata-bearing land plants, and the recent evolutionary origins of some *Arabidopsis* components by gene duplication (e.g., *SPCH*, *MUTE*, *ERECTA*, and *YODA*), raise the possibility that uncharacterized genes in bryophytes and early-diverging tracheophytes may play important roles in the development and function of stomata and may have contributed to stomatal origins and early evolution.

The presence of orthologs of genes regulating stomatal development and function in liverworts provides further evidence for the hypothesis that stomata pre-dated the split between bryophytes and tracheophytes (Figure 3A) and that liverworts are descended from stomata-bearing ancestors [2, 40]. Extant early-diverging liverworts, such as *Treubia lacunose*, lack both stomata and air pores [41], suggesting that air pores evolved later during liverwort diversification. That is, stomata were lost in the liverwort lineage after its divergence from other bryophytes, the common ancestor of modern liverworts possessed neither stomata nor air pores, and air pores evolved subsequently during the diversification of liverworts [43]. Cumulatively, these analyses suggest that the liverwort air pore is distinct from stomata in terms of both evolutionary origin and function. It is possible that, due to the moist environment favored by extant liverworts, the need to actively regulate water loss was reduced. In this scenario, the selective pressure to retain energetically expensive guard cells [44] would be relaxed. The evolution of the static air pore in more derived liverworts [43, 45] might have provided a conduit for gas exchange without the unnecessary and costly active control of guard cell turgor pressure.

An alternative scenario for the origin of the liverwort air pore is that they evolved to facilitate interaction with prokaryotic symbionts. Work by Alcaraz and colleagues showed that the air pores of the *Marchantia* harbor methylotrophic bacteria [46]. Interestingly, liverworts, such as *Blasia*, which lack both air pores and stomata, have dome-shaped structures known as auricles on

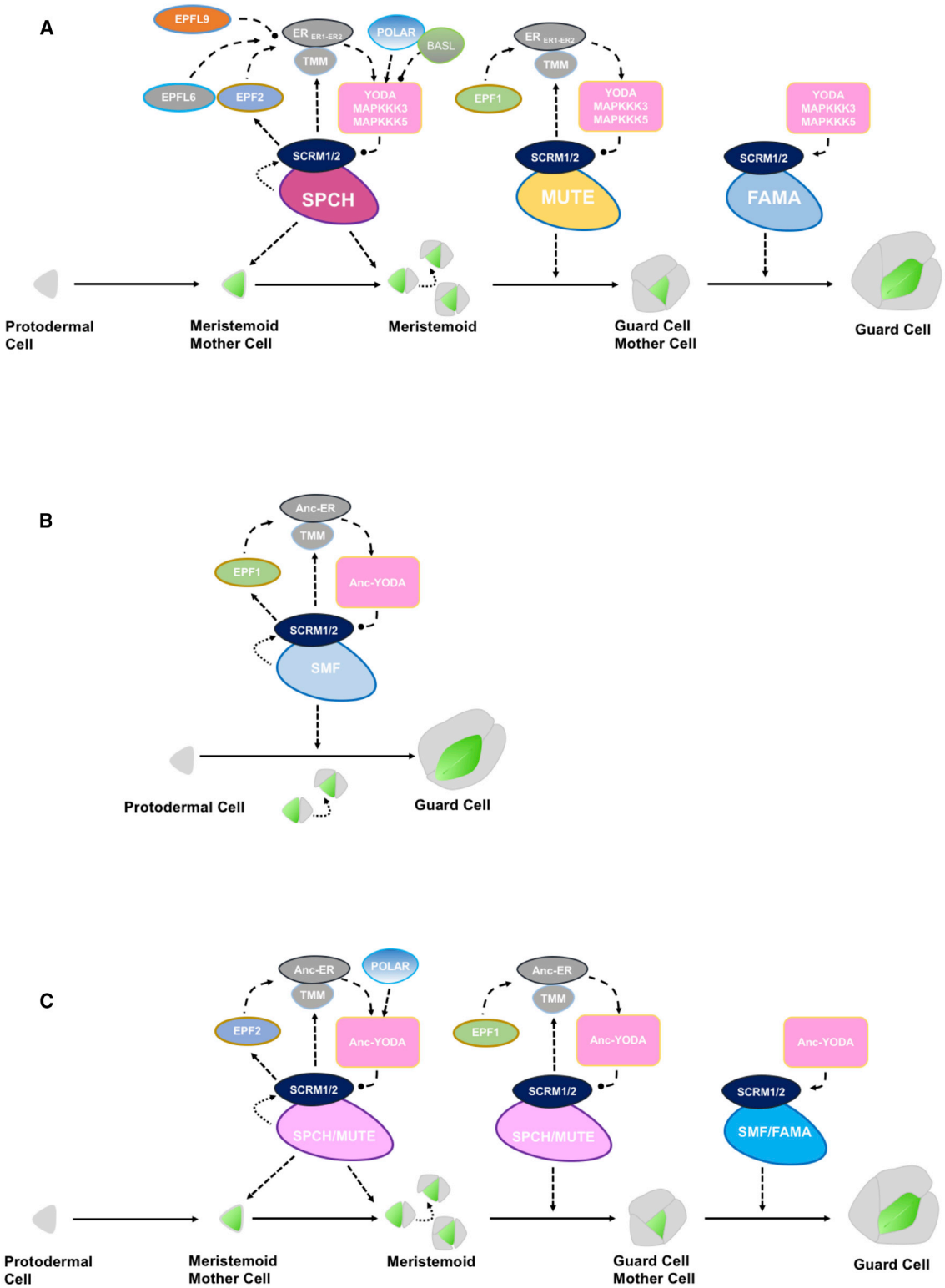


Figure 6. Stomatal Development Pathways for *Arabidopsis thaliana* and *Physcomitrella patens* and Reconstruction of the Ancestral Stomataphyte Pathway

(A) Stomatal developmental pathway in *Arabidopsis thaliana*—figure adapted from [42].

(B) Hypothesized stomatal developmental pathway of *Physcomitrella* was created by analyzing the presence of orthologs shared with the *Arabidopsis* developmental pathway (Figure 3A).

(legend continued on next page)

the ventral surface, which are occupied by cyanobacterial colonies [47]. Further investigation into the genetic underpinnings of the auricle and the air pore may provide insights into the evolution and function of these structures.

In summary, our analyses suggest that stomata are a homologous, evolutionarily ancient structure that evolved once in the common ancestor of all land plants. The stomata of early embryophytes were morphologically and functionally more sophisticated than previously envisioned, and bryophyte stomata underwent reductive evolution. Key developmental and functional genes were lost in mosses and hornworts, and the structures were entirely lost from the last common ancestor of modern liverworts and some early-diverging mosses. The results suggest that the liverwort air pore evolved subsequent to stomatal loss, likely for a different function.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Data and Code Availability
  - Materials Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Sequence data
  - Guard cell toolkit
- METHOD DETAILS
  - Comparison of manual curated orthology groups and automated approach (Orthofinder)
  - Phylogenetic analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.03.048.

ACKNOWLEDGMENTS

B.J.H. is grateful to the New Phytologist Trust for the award of a PhD studentship. A.M.H. acknowledges support from the New Phytologist Trust. T.A.W. is supported by a Royal Society University Research Fellowship. A.M.H. and T.A.W. are grateful to the Leverhulme Trust for the award of a Research Project Grant.

AUTHOR CONTRIBUTIONS

B.J.H. performed the experiments. T.A.W., A.M.H., C.J.H., and B.J.H. conceived and designed the experiments. B.J.H. and T.A.W. wrote the manuscript with input from A.M.H. and C.J.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: November 13, 2019
Revised: February 13, 2020
Accepted: March 18, 2020
Published: April 16, 2020

REFERENCES

1. Pires, N.D., and Dolan, L. (2012). Morphological evolution in land plants: new designs with old genes. Philos. Trans. R. Soc. Lond. B Biol. Sci. *367*, 508–518.
2. Puttick, M.N., Morris, J.L., Williams, T.A., Cox, C.J., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Schneider, H., Pisani, D., and Donoghue, P.C.J. (2018). The interrelationships of land plants and the nature of the ancestral embryophyte. Curr. Biol. *28*, 733–745.e2.
3. Morris, J.L., Puttick, M.N., Clark, J.W., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Yang, Z., Schneider, H., and Donoghue, P.C.J. (2018). The timescale of early land plant evolution. Proc. Natl. Acad. Sci. USA *115*, E2274–E2283.
4. de Vries, J., and Archibald, J.M. (2018). Plant evolution: landmarks on the path to terrestrial life. New Phytol. *217*, 1428–1434.
5. Donoghue, P., and Paps, J. (2020). Plant evolution: assembling land plants. Curr. Biol. *30*, R81–R83.
6. Jill Harrison, C. (2017). Development and genetics in the evolution of land plant body plans. Philos. Trans. R. Soc. Lond. B Biol. Sci. *372*, 20150490.
7. Harrison, C.J., and Morris, J.L. (2018). The origin and early evolution of vascular plant shoots and leaves. Philos. Trans. R. Soc. Lond. B Biol. Sci. *373*, 20160496.
8. Raven, J.A. (2002). Selection pressures on stomatal evolution. New Phytol. *153*, 371–386.
9. Chater, C.C., Caine, R.S., Tomek, M., Wallace, S., Kamisugi, Y., Cuming, A.C., Lang, D., MacAlister, C.A., Casson, S., Bergmann, D.C., et al. (2016). Origin and function of stomata in the moss Physcomitrella patens. Nat. Plants *2*, 16179.
10. Pressel, S., Renzaglia, K.S., Dicky Clymo, R.S., and Duckett, J.G. (2018). Hornwort stomata do not respond actively to exogenous and environmental cues. Ann. Bot. *122*, 45–57.
11. Duckett, J.G., and Pressel, S. (2018). The evolution of the stomatal apparatus: intercellular spaces and sporophyte water relations in bryophytes-two ignored dimensions. Philos. Trans. R. Soc. Lond. B Biol. Sci. *373*, 20160498.
12. Cox, C.J. (2018). Land plant molecular phylogenetics: a review with comments on evaluating incongruence among phylogenies. Crit. Rev. Plant Sci. *37*, 113–127.
13. Merced, A., and Renzaglia, K. (2014). Developmental changes in guard cell wall structure and pectin composition in the moss Funaria: implications for function and evolution of stomata. Ann. Bot. *114*, 1001–1010.
14. Cullen, E., and Rudall, P.J. (2016). The remarkable stomata of horsetails (Equisetum): patterning, ultrastructure and development. Ann. Bot. *118*, 207–218.
15. Pillitteri, L.J., and Dong, J. (2013). Stomatal development in Arabidopsis. Arabidopsis Book *11*, e0162.

(C) Ancestral reconstruction was based on the orthologs inferred to be ancestrally present in the common ancestor of bryophytes and tracheophytes, as presented in Figure 3A. The phylogenies suggest that EPF1/2, SMF, and a protein ancestral to both SPCH and MUTE were present in the ancestral stomataphyte and that the asymmetric amplification of cells surrounding stomata (stomatal-lineage ground cells, depicted in gray) and stomatal spacing was characteristic of ancestral stomataphytes. The reconstruction suggests that ancestral stomataphytes were more similar to extant tracheophytes than bryophytes.
In (B) and (C), Anc-*YODA* is an ortholog of three *Arabidopsis* genes: *YODA*; *MAPKKK3*; and *MAPKKK5*. These three modern proteins show functional redundancy (Data S1A), and so we hypothesize that *Arabidopsis* YODA functions are fulfilled by Anc-YODA in *Physcomitrella* and that these functions are ancestral. Similarly, *Anc-ER* is an ortholog of *Arabidopsis ERECTA*, *ERL-1*, and *ERL-2*. In (B), note only partial one-cell spacing occurring in *Physcomitrella*, i.e., there is only partial amplification of protodermal cells to ensure stomata are spaced, but not equally on the epidermis [31].

16. Mishler, B.D., and Churchill, S.P. (1985). Transition to a land flora: phylogenetic relationships of the green algae and bryophytes. Cladistics *1*, 305–328.
17. Renzaglia, K.S., and Garbary, D.J. (2001). Motile gametes of land plants: diversity, development, and evolution. Crit. Rev. Plant Sci. *20*, 107–213.
18. Cox, C.J., Li, B., Foster, P.G., Embley, T.M., and Civán, P. (2014). Conflicting phylogenies for early land plants are caused by composition biases among synonymous substitutions. Syst. Biol. *63*, 272–279.
19. Leebens-Mack, J.H., Barker, M.S., Carpenter, E.J., Deyholos, M.K., Gitzendanner, M.A., Graham, S.W., Grosse, I., Li, Z., Melkonian, M., Mirarab, S., et al.; One Thousand Plant Transcriptomes Initiative (2019). One thousand plant transcriptomes and the phylogenomics of green plants. Nature *574*, 679–685.
20. Chater, C., Gray, J.E., and Beerling, D.J. (2013). Early evolutionary acquisition of stomatal control and development gene signalling networks. Curr. Opin. Plant Biol. *16*, 638–646.
21. Chater, C.C.C., Caine, R.S., Fleming, A.J., and Gray, J.E. (2017). Origins and evolution of stomatal development. Plant Physiol. *174*, 624–638.
22. Pressel, S., Goral, T., and Duckett, J.G. (2014). Stomatal differentiation and abnormal stomata in hornworts. J. Bryol. *36*, 87–103.
23. de Sousa, F., Foster, P.G., Donoghue, P.C.J., Schneider, H., and Cox, C.J. (2019). Nuclear protein phylogenies support the monophyly of the three bryophyte groups (Bryophyta Schimp.). New Phytol. *222*, 565–575.
24. Emms, D.M., and Kelly, S. (2019). OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. *20*, 238.
25. Nguyen, L.T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. *32*, 268–274.
26. Bates, G.W., Rosenthal, D.M., Sun, J., Chattopadhyay, M., Peffer, E., Yang, J., Ort, D.R., and Jones, A.M. (2012). A comparative study of the Arabidopsis thaliana guard-cell transcriptome and its modulation by sucrose. PLoS ONE *7*, e49641.
27. Bauer, H., Ache, P., Lautner, S., Fromm, J., Hartung, W., Al-Rasheid, K.A.S., Sonnewald, S., Sonnewald, U., Kneitz, S., Lachmann, N., et al. (2013). The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr. Biol. *23*, 53–57.
28. Ohno, S. (1970). Evolution by Gene Duplication (Springer).
29. Fitch, W.M. (1970). Distinguishing homologous from analogous proteins. Syst. Zool. *19*, 99–113.
30. Altenhoff, A.M., Studer, R.A., Robinson-Rechavi, M., and Dessimoz, C. (2012). Resolving the ortholog conjecture: orthologs tend to be weakly, but significantly, more similar in function than paralogs. PLoS Comput. Biol. *8*, e1002514.
31. Caine, R.S., Chater, C.C., Kamisugi, Y., Cuming, A.C., Beerling, D.J., Gray, J.E., and Fleming, A.J. (2016). An ancestral stomatal patterning module revealed in the non-vascular land plant Physcomitrella patens. Development *143*, 3306–3314.
32. Kurbidaeva, A., Ezhova, T., and Novokreshchenova, M. (2014). Arabidopsis thaliana ICE2 gene: phylogeny, structural evolution and functional diversification from ICE1. Plant Sci. *229*, 10–22.
33. Miura, K., Ohta, M., Nakazawa, M., Ono, M., and Hasegawa, P.M. (2011). ICE1 Ser403 is necessary for protein stabilization and regulation of cold signaling and tolerance. Plant J. *67*, 269–279.
34. Kidokoro, S., Kim, J.-S., Ishikawa, T., Suzuki, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2020). *DREB1A/CBF3* is repressed by transgene-induced DNA methylation in the Arabidopsis *ice1-1* mutant. Plant Cell, tpc.00532.2019.
35. Olsen, J.L., Rouzé, P., Verhelst, B., Lin, Y.C., Bayer, T., Collen, J., Dattolo, E., De Paoli, E., Dittami, S., Maumus, F., et al. (2016). The genome of the seagrass Zostera marina reveals angiosperm adaptation to the sea. Nature *530*, 331–335.
36. O'Donoghue, M.T., Chater, C., Wallace, S., Gray, J.E., Beerling, D.J., and Fleming, A.J. (2013). Genome-wide transcriptomic analysis of the sporophyte of the moss Physcomitrella patens. J. Exp. Bot. *64*, 3567–3581.
37. Sussmilch, F.C., Schultz, J., Hedrich, R., and Roelfsema, M.R.G. (2019). Acquiring control: the evolution of stomatal signalling pathways. Trends Plant Sci. *24*, 342–351.
38. Takemiya, A., Sugiyama, N., Fujimoto, H., Tsutsumi, T., Yamauchi, S., Hiyama, A., Tada, Y., Christie, J.M., and Shimazaki, K. (2013). Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. Nat. Commun. *4*, 2094.
39. Komatsu, A., Terai, M., Ishizaki, K., Suetsugu, N., Tsuboi, H., Nishihama, R., Yamato, K.T., Wada, M., and Kohchi, T. (2014). Phototropin encoded by a single-copy gene mediates chloroplast photorelocation movements in the liverwort Marchantia polymorpha. Plant Physiol. *166*, 411–427.
40. Wickett, N.J., Mirarab, S., Nguyen, N., Warnow, T., Carpenter, E., Matasci, N., Ayyampalayam, S., Barker, M.S., Burleigh, J.G., Gitzendanner, M.A., et al. (2014). Phylotranscriptomic analysis of the origin and early diversification of land plants. Proc. Natl. Acad. Sci. USA *111*, E4859–E4868.
41. Sachs, T. (1991). Pattern Formation in Plant Tissues (Cambridge University).
42. Han, S.K., and Torii, K.U. (2016). Lineage-specific stem cells, signals and asymmetries during stomatal development. Development *143*, 1259–1270.
43. Villarreal A, J.C., Crandall-Stotler, B.J., Hart, M.L., Long, D.G., and Forrest, L.L. (2016). Divergence times and the evolution of morphological complexity in an early land plant lineage (Marchantiopsida) with a slow molecular rate. New Phytol. *209*, 1734–1746.
44. Haworth, M., Elliott-Kingston, C., and McElwain, J.C. (2011). Stomatal control as a driver of plant evolution. J. Exp. Bot. *62*, 2419–2423.
45. Shimamura, M. (2016). Marchantia polymorpha: taxonomy, phylogeny and morphology of a model system. Plant Cell Physiol. *57*, 230–256.
46. Alcaraz, L.D., Peimbert, M., Barajas, H.R., Dorantes-Acosta, A.E., Bowman, J.L., and Arteaga-Vázquez, M.A. (2018). Marchantia liverworts as a proxy to plants' basal microbiomes. Sci. Rep. *8*, 12712.
47. Adams, D.G., and Duggan, P.S. (2008). Cyanobacteria-bryophyte symbioses. J. Exp. Bot. *59*, 1047–1058.
48. Matasci, N., Hung, L.H., Yan, Z., Carpenter, E.J., Wickett, N.J., Mirarab, S., Nguyen, N., Warnow, T., Ayyampalayam, S., Barker, M., et al. (2014). Data access for the 1,000 Plants (1KP) project. Gigascience *3*, 17.
49. Katoh, K., and Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. Brief. Bioinform. *9*, 286–298.
50. Criscuolo, A., and Gribaldo, S. (2010). BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC Evol. Biol. *10*, 210.
51. Letunic, I., and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics *23*, 127–128.
52. Johnson, M.T.J., Carpenter, E.J., Tian, Z., Bruskiewich, R., Burris, J.N., Carrigan, C.T., Chase, M.W., Clarke, N.D., Covshoff, S., Depamphilis, C.W., et al. (2012). Evaluating methods for isolating total RNA and predicting the success of sequencing phylogenetically diverse plant transcriptomes. PLoS ONE *7*, e50226.
53. Xie, Y., Wu, G., Tang, J., Luo, R., Patterson, J., Liu, S., Huang, W., He, G., Gu, S., Li, S., et al. (2014). SOAPdenovo-Trans: de novo transcriptome assembly with short RNA-seq reads. Bioinformatics *30*, 1660–1666.
54. Munemasa, S., Hauser, F., Park, J., Waadt, R., Brandt, B., and Schroeder, J.I. (2015). Mechanisms of abscisic acid-mediated control of stomatal aperture. Curr. Opin. Plant Biol. *28*, 154–162.
55. Feijó, J.A., and Wudick, M.M. (2018). Calcium is life. J. Exp. Bot. *69*, 4147–4150.
56. Ache, P., Becker, D., Ivashikina, N., Dietrich, P., Roelfsema, M.R.G., and Hedrich, R. (2000). GORK, a delayed outward rectifier expressed in guard cells of Arabidopsis thaliana, is a K(+)-selective, K(+)-sensing ion channel. FEBS Lett. *486*, 93–98.
57. Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M., and Iba, K. (2008). CO2

regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature *452*, 483–486.

58. Rusconi, F., Simeoni, F., Francia, P., Cominelli, E., Conti, L., Riboni, M., Simoni, L., Martin, C.R., Tonelli, C., and Galbiati, M. (2013). The Arabidopsis thaliana MYB60 promoter provides a tool for the spatio-temporal control of gene expression in stomatal guard cells. J. Exp. Bot. *64*, 3361–3371.
59. Padmanaban, S., Chanroj, S., Kwak, J.M., Li, X., Ward, J.M., and Sze, H. (2007). Participation of endomembrane cation/H+ exchanger AtCHX20 in osmoregulation of guard cells. Plant Physiol. *144*, 82–93.
60. Hashimoto, M., Negi, J., Young, J., Israelsson, M., Schroeder, J.I., and Iba, K. (2006). Arabidopsis HT1 kinase controls stomatal movements in response to CO2. Nat. Cell Biol. *8*, 391–397.
61. Galbiati, M., Simoni, L., Pavesi, G., Cominelli, E., Francia, P., Vavasseur, A., Nelson, T., Bevan, M., and Tonelli, C. (2008). Gene trap lines identify Arabidopsis genes expressed in stomatal guard cells. Plant J. *53*, 750–762.
62. Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J. (2002). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell *14*, 3089–3099.
63. Kwak, J.M., Murata, Y., Baizabal-Aguirre, V.M., Merrill, J., Wang, M., Kemper, A., Hawke, S.D., Tallman, G., and Schroeder, J.I. (2001). Dominant negative guard cell K+ channel mutants reduce inward-rectifying K+ currents and light-induced stomatal opening in arabidopsis. Plant Physiol. *127*, 473–485.
64. Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K. (2001). Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature *414*, 656–660.
65. Stadler, R., Büttner, M., Ache, P., Hedrich, R., Ivashikina, N., Melzer, M., Shearson, S.M., Smith, S.M., and Sauer, N. (2003). Diurnal and light-regulated expression of AtSTP1 in guard cells of Arabidopsis. Plant Physiol. *133*, 528–537.
66. Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y., and Schroeder, J.I. (1999). Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. Plant Cell *11*, 1785–1798.
67. Desikan, R., Last, K., Harrett-Williams, R., Tagliavia, C., Harter, K., Hooley, R., Hancock, J.T., and Neill, S.J. (2006). Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. Plant J. *47*, 907–916.
68. Klein, M., Perfus-Barbeoch, L., Frelet, A., Gaedeke, N., Reinhardt, D., Mueller-Roeber, B., Martinoia, E., and Forestier, C. (2003). The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use. Plant J. *33*, 119–129.
69. Gobert, A., Isayenkov, S., Voelker, C., Czempinski, K., and Maathuis, F.J.M. (2007). The two-pore channel TPK1 gene encodes the vacuolar K+ conductance and plays a role in K+ homeostasis. Proc. Natl. Acad. Sci. USA *104*, 10726–10731.
70. Kim, J.S., Jung, H.J., Lee, H.J., Kim, K.A., Goh, C.H., Woo, Y., Oh, S.H., Han, Y.S., and Kang, H. (2008). Glycine-rich RNA-binding protein 7 affects abiotic stress responses by regulating stomata opening and closing in Arabidopsis thaliana. Plant J. *55*, 455–466.
71. Hubbard, K.E., Siegel, R.S., Valerio, G., Brandt, B., and Schroeder, J.I. (2012). Abscisic acid and CO2 signalling via calcium sensitivity priming in guard cells, new CDPK mutant phenotypes and a method for improved resolution of stomatal stimulus-response analyses. Ann. Bot. *109*, 5–17.
72. Ohashi-Ito, K., and Bergmann, D.C. (2006). Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell *18*, 2493–2505.
73. Kanaoka, M.M., Pillitteri, L.J., Fujii, H., Yoshida, Y., Bogenschutz, N.L., Takabayashi, J., Zhu, J.K., and Torii, K.U. (2008). SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to arabidopsis stomatal differentiation. Plant Cell *20*, 1775–1785.
74. Yang, M., and Sack, F.D. (1995). The too many mouths and four lips mutations affect stomatal production in Arabidopsis. Plant Cell *7*, 2227–2239.
75. Masle, J., Gilmore, S.R., and Farquhar, G.D. (2005). The ERECTA gene regulates plant transpiration efficiency in Arabidopsis. Nature *436*, 866–870.
76. Hara, K., Kajita, R., Torii, K.U., Bergmann, D.C., and Kakimoto, T. (2007). The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Genes Dev. *21*, 1720–1725.
77. Hunt, L., and Gray, J.E. (2009). The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. Curr. Biol. *19*, 864–869.
78. Lin, G., Zhang, L., Han, Z., Yang, X., Liu, W., Li, E., Chang, J., Qi, Y., Shpak, E.D., and Chai, J. (2017). A receptor-like protein acts as a specificity switch for the regulation of stomatal development. Genes Dev. *31*, 927–938.
79. Pillitteri, L.J., Bogenschutz, N.L., and Torii, K.U. (2008). The bHLH protein, MUTE, controls differentiation of stomata and the hydathode pore in Arabidopsis. Plant Cell Physiol. *49*, 934–943.
80. MacAlister, C.A., Ohashi-Ito, K., and Bergmann, D.C. (2007). Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature *445*, 537–540.
81. Dong, J., MacAlister, C.A., and Bergmann, D.C. (2009). BASL controls asymmetric cell division in Arabidopsis. Cell *137*, 1320–1330.
82. Bergmann, D.C., Lukowitz, W., and Somerville, C.R. (2004). Stomatal development and pattern controlled by a MAPKK kinase. Science *304*, 1494–1497.
83. Pillitteri, L.J., Peterson, K.M., Horst, R.J., and Torii, K.U. (2011). Molecular profiling of stomatal meristemoids reveals new component of asymmetric cell division and commonalities among stem cell populations in Arabidopsis. Plant Cell *23*, 3260–3275.
84. Jonak, C., Okrész, L., Bögre, L., and Hirt, H. (2002). Complexity, cross talk and integration of plant MAP kinase signalling. Curr. Opin. Plant Biol. *5*, 415–424.
85. Zhao, X., Wang, Y.L., Qiao, X.R., Wang, J., Wang, L.D., Xu, C.S., and Zhang, X. (2013). Phototropins function in high-intensity blue light-induced hypocotyl phototropism in Arabidopsis by altering cytosolic calcium. Plant Physiol. *162*, 1539–1551.
86. Rubio, S., Rodrigues, A., Saez, A., Dizon, M.B., Galle, A., Kim, T.H., Santiago, J., Flexas, J., Schroeder, J.I., and Rodriguez, P.L. (2009). Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. Plant Physiol. *150*, 1345–1355.
87. Yang, Z., Liu, J., Poree, F., Schaeufele, R., Helmke, H., Frackenpohl, J., Lehr, S., von Koskull-Döring, P., Christmann, A., Schnyder, H., et al. (2019). Abscisic acid receptors and coreceptors modulate plant water use efficiency and water productivity. Plant Physiol. *180*, 1066–1080.
88. Wang, Y.F., Munemasa, S., Nishimura, N., Ren, H.M., Robert, N., Han, M., Puzõrjova, I., Kollist, H., Lee, S., Mori, I., and Schroeder, J.I. (2013). Identification of cyclic GMP-activated nonselective Ca2+-permeable cation channels and associated CNGC5 and CNGC6 genes in Arabidopsis guard cells. Plant Physiol. *163*, 578–590.
89. Tunc-Ozdemir, M., Rato, C., Brown, E., Rogers, S., Mooneyham, A., Frietsch, S., Myers, C.T., Poulsen, L.R., Malhó, R., and Harper, J.F. (2013). Cyclic nucleotide gated channels 7 and 8 are essential for male reproductive fertility. PLoS ONE *8*, e55277.
90. Brost, C., Studtrucker, T., Reimann, R., Denninger, P., Czekalla, J., Krebs, M., Fabry, B., Schumacher, K., Grossmann, G., and Dietrich, P. (2019). Multiple cyclic nucleotide-gated channels coordinate calcium oscillations and polar growth of root hairs. Plant J. *99*, 910–923.
91. Jin, Y., Jing, W., Zhang, Q., and Zhang, W. (2015). Cyclic nucleotide gated channel 10 negatively regulates salt tolerance by mediating Na+ transport in Arabidopsis. J. Plant Res. *128*, 211–220.
92. Moon, J.Y., Belloeil, C., Ianna, M.L., and Shin, R. (2019). Arabidopsis CNGC family members contribute to heavy metal ion uptake in plants. Int. J. Mol. Sci. *20*, E413.

93. Gao, Q.F., Gu, L.L., Wang, H.Q., Fei, C.F., Fang, X., Hussain, J., Sun, S.J., Dong, J.Y., Liu, H., and Wang, Y.F. (2016). Cyclic nucleotide-gated channel 18 is an essential Ca2+ channel in pollen tube tips for pollen tube guidance to ovules in Arabidopsis. Proc. Natl. Acad. Sci. USA *113*, 3096–3101.

94. Ishida, K., Yamashino, T., Yokoyama, A., and Mizuno, T. (2008). Three type-B response regulators, ARR1, ARR10 and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of Arabidopsis thaliana. Plant Cell Physiol. *49*, 47–57.

95. Lu, Y., Chanroj, S., Zulkifli, L., Johnson, M.A., Uozumi, N., Cheung, A., and Sze, H. (2011). Pollen tubes lacking a pair of K+ transporters fail to target ovules in Arabidopsis. Plant Cell *23*, 81–93.

96. Sun, T., Nitta, Y., Zhang, Q., Wu, D., Tian, H., Lee, J.S., and Zhang, Y. (2018). Antagonistic interactions between two MAP kinase cascades in plant development and immune signaling. EMBO Rep. *19*, e45324.

97. Qu, X., Zhao, Z., and Tian, Z. (2017). ERECTA regulates cell elongation by activating auxin biosynthesis in arabidopsis thaliana. Front. Plant Sci. *8*, 1688.

98. Minh, B.Q., Nguyen, M.A.T., and von Haeseler, A. (2013). Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. *30*, 1188–1195.

99. Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. Syst. Biol. *51*, 492–508.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
OneKP project	[48]	https://sites.google.com/a/ualberta.ca/onekp/
NCBI	NCBI website	https://www.ncbi.nlm.nih.gov/genome
Raw and analyzed data	This study	https://figshare.com/authors/Brogan_Harris/7859150
Software and Algorithms		
MAFFT	[49]	https://mafft.cbrc.jp/alignment/software/
BMGE 4.0	[50]	https://omictools.com/bmge-tool
IQ-Tree	[25]	http://www.iqtree.org/
OrthoFinder 2.0	[24]	https://github.com/davidemms/OrthoFinder/releases
ITOL	[51]	https://itol.embl.de/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tom Williams (tom.a.williams@bristol.ac.uk).

Data and Code Availability

All proteome and transcriptome data was downloaded from NCBI and the 1KP project [40, 48, 52, 53] respectively. All query sequences, multiple sequence alignments and Newick tree files are available on FigShare https://doi.org/10.6084/m9.figshare.10298117, 10.6084/m9.figshare.10298093 and 0.6084/m9.figshare.10298078.v1 respectively. Custom Python scripts that were used to handle sequence data and automate BLAST searches have been made available at https://github.com/Brogan-Harris/Phylogenomics.

Materials Availability

There are no materials to report.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sequence data

A local dataset was compiled from proteomes downloaded from NCBI and transcriptomes from the 1KP project [40, 48, 52, 53]. The dataset consisted of amino acid sequence data from 177 species of plants and green algae (Data S1G).

Guard cell toolkit

We defined the guard cell toolkit by reference to published mutant phenotypes, functional analyses and gene expression data. For genes whose products are involved in stomatal function in *Arabidopsis*, we required both (i) greater expression in guard cells than in pavement cells and (ii) a published mutant phenotype. Our toolkit therefore represents a conservative, high-confidence estimate of the suite of genes that specify guard cell development and function in *Arabidopsis.* The expression levels of guard cell genes were identified from data published by Bauer et al., and Bates et al. [26, 27]. These genes were then cross-referenced with molecular studies to ensure that they were implicated in guard cell function. Genes involved in stomatal development are more difficult to identify because they are not necessarily highly expressed in mature guard cells, and expression data is not always available for the relevant developmental stage. Therefore, genes whose products are involved in stomatal development were identified by review of the literature on guard cell development. The assembled toolkit and the associated references [54–97] are presented in Data S1I.

METHOD DETAILS

Comparison of manual curated orthology groups and automated approach (Orthofinder)

To complement our manually curated gene tree and ortholog inferences, we repeated our analyses using the automated ortholog inference method OrthoFinder [24]. Where families could be directly compared, the results agreed (Data S1A–S1E). However, the OrthoFinder analysis was unable to resolve orthology and paralogy relationships among 10 of the functional genes and one developmental gene, *YODA*, that are part of large multigene families in plants, returning very large families (> 1000 members) whose

histories are difficult to interpret because sequence alignment and tree inference is challenging (Data S1B and S1E). We therefore focus on the manually curated datasets in the results section.

Phylogenetic analysis

Species tree inference

151 single copy orthologs were identified with Orthofinder [24]; in-paralogs were removed with a custom python script (https://github.com/Brogan-Harris/Phylogenomics). The single copy orthologs were aligned using MAFFT [49] and trimmed with BMGE 4.0 with a BLOSUM40 matrix [50]. The 151 multiple sequence alignments were then concatenated into a super matrix using a custom python code (https://github.com/Brogan-Harris/Phylogenomics). A bootstrapped maximum likelihood phylogeny was inferred in IQ-Tree [25], using BIC to select the best-fitting substitution model (LG+C60+G+F; as site heterogeneity is a pervasive feature of plant sequence evolution [2]), and empirical profile mixture models (C10-C60), which model site-specific biochemical constraints and often improve model fit, were included in the model search. The tree was rooted in accordance with previous published studies [2, 4, 5, 40].

Gene tree inference

BLAST searches for toolkit components using an e-value cut-off of $1e^{-20}$ were undertaken on the local dataset described in the 'Sequence data' section. Homologous sequences were aligned using MAFFT [49]. The multiple sequence alignments were then trimmed using BMGE 4.0 with a BLOSUM30 matrix [50] to identify and remove poorly aligning positions. Bootstrapped maximum likelihood phylogenies were inferred in IQ-Tree [25], using BIC to select the best-fitting substitution model, and as site heterogeneity is a pervasive feature of plant sequence evolution [2], empirical profile mixture models (C10-C60), which model site-specific biochemical constraints and often improve model fit, were included in the model search. Trees were rooted in accordance with the most up to date species tree [2, 19], and the species tree presented in Figure 1 to infer gene origins, duplications and losses of analyzed genes. Trees were visualized and edited in ITOL [51].

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequence similarity was quantified using BLASTP E-values. Best-fitting phylogenetic models were selected according to the Bayesian Information Criterion implemented in IQ-Tree ([25]). Branch supports were estimated using UFBoot2 [98] bootstrapping in IQ-Tree. We used approximately-unbiased (AU) tests [99] to compare support for the scenarios of gene family evolution discussed in the text, using the maximum likelihood tree that satisfied the relevant topological constraint for comparison in each case.